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Clostridium perfringens enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein

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Abstract Claudins (claudin-1 to -18) with four transmembrane domains and two extracellular loops constitute tight junction strands. The peptide toxin *Clostridium perfringens* enterotoxin (CPE) has been shown to bind to claudin-3 and -4, but not to claudin-1 or -2. We constructed claudin-1/claudin-3 chimeric molecules and found that the second extracellular loop of claudin-3 conferred CPE sensitivity on L fibroblasts. Furthermore, overlay analyses revealed that the second extracellular loop of claudin-3 specifically bound to CPE at the K_a value of 1.0×10^8 M^{-1}. We concluded that the second extracellular loop is the site through which claudin-3 interacts with CPE on the cell surface.

Key words: Claudin; Tight junction; Tight junction strand; Barrier; *Clostridium perfringens* enterotoxin

1. Introduction

The tight junction (TJ) is one mode of cell-to-cell adhesion in epithelial and endothelial cellular sheets, which is located at the most apical part of their lateral membranes [1]. TJs seal the cells to create a primary barrier to the diffusion of solutes across the cellular sheet and also function as a boundary between the apical and basolateral membrane domains to produce their polarization [2–4]. Both the barrier and fence functions of TJs are essential for multicellular organisms. On ultrathin section electron microscopy, TJs appear as a series of discrete sites of apparent fusion, involving the outer leaflet of the plasma membranes of adjacent cells [1]. On freeze-fracture electron microscopy, TJs appear as a set of continuous, anastomosing intramembraneous particle strands (TJ strands) [5].

Two related integral membrane proteins with molecular masses of ~23 kDa, claudin-1 and -2, have been identified as major constituents of TJ strands [6]. When claudin-1 or -2 was singly introduced into mouse L fibroblasts lacking TJs, well developed networks of TJ strands were reconstituted between adjacent transfectants [7]. These claudin molecules are composed of four transmembrane domains and two extracellular (EC) loops with both NH_2- and COOH-termini in the cytoplasm. To date, 18 distinct species of claudins (claudin-1 to -18) have been identified [8–10]. Among these, claudin-4 was initially identified as CPE-R, a cellular receptor for *Clostridium perfringens* enterotoxin (CPE) [11]. This enterotoxin, which consists of a single polypeptide chain with a molecular mass of ~35 kDa, is the causative agent of symptoms associated with *Clostridium perfringens* food poisoning in man [12]. In addition to claudin-4/CPE-R, claudin-3, which was initially identified as RVP1 (rat ventral prostate-1) [13], was also reported to function as a receptor for CPE [14]. Furthermore, it is now accepted that the COOH-terminal half of this toxin (C-CPE) binds to claudin-4/CPE-R, and that its NH_2-terminal half increases membrane permeability by forming small pores in the plasma membrane [15,16].

Recently, we found that incubation with C-CPE resulted in the specific removal of claudin-4 from TJs of MDCK I cells that mainly express claudin-1 and -4 [17]. Concomitantly, the number of TJ strands as well as the barrier function of TJs (transepithelial resistance) were significantly decreased. These findings revealed first that claudins are directly involved in the barrier function of TJs. Furthermore, these findings suggested that polypeptides such as C-CPE can be utilized to modulate the TJ barrier at the whole body level as well as at the cellular level, which would contribute significantly to the development of methods for drug delivery. In this study, as a step along this line, we identified the region on claudin-3 that is responsible for its specific binding to C-CPE.

2. Materials and methods

2.1. Antibodies and cells

Rat anti-mouse claudin-1 monoclonal antibody (mAb) and rabbit anti-mouse claudin-3 polyclonal antibody (pAb) were raised and characterized previously [8,18]. Rabbit anti-CPE pAb was raised against recombinant CPE produced in *Escherichia coli* [19].

L transfectants expressing claudin-1 (C1L) and claudin-3 (C3L) were established previously [18]. L transfectants were cultured on coverslips in DMEM supplemented with 10% fetal calf serum.

2.2. Establishment of L transfectants expressing claudin-1/claudin-3 chimeras

As shown in Fig. 1, two types of chimeric molecules between claudin-1 and claudin-3 were constructed: cDNA fragments encoding

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Abbreviations: CPE, *Clostridium perfringens* enterotoxin; CPE-R, CPE receptor; C-CPE, COOH-terminal half of CPE; EC loop, extracellular loop; TJ, tight junction; mAb, monoclonal antibody; pAb, polyclonal antibody

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amino acids (aa) 1–104 of claudin-1, aa 105–211 of claudin-1, aa 1–103 of claudin-3 or aa 104–219 of claudin-3 were amplified by polymerase chain reaction, and cDNA fragments encoding claudin-1/3 or claudin-3/1 were subcloned into pBluescript SK (−). Inserts were excised by *Eco*RI digestion, blunted with T4 polymerase and ligated into pCAGGSneodel*Eco*RI [20], which was provided by Dr. J. Miyazaki (Osaka University). Mouse L fibroblasts were transfected with these expression vectors, and L transfectants stably expressing claudin-1/3 or -3/1 (C1/3L or C3/1L cells, respectively) were established according to the methods described previously [7,18]. Several stable clones were isolated for each transfection experiment.

2.3. Production of CPE/C-CPE and cytotoxicity assay

CPE was purified by the method of Sakaguchi et al. [21]. The COOH-terminal fragment (184–319 aa) of CPE with a 10-histidine tag was produced in *E. coli* and purified as described previously [11]. The cytotoxic effect of CPE on L transfectants was determined by examining their morphological alterations 24 h after the addition of CPE (500 ng/ml) to the culture medium. The binding of ^{125}I-CPE to L transfectants expressing the respective claudins was measured and Scatchard analysis was performed as described previously [11,17].

2.4. Production of glutathione S-transferase (GST) fusion proteins in E. coli

The cDNA fragments encoding the first EC loop of claudin-3 (aa 30–81), the second EC loop of claudin-3 (aa 143–160) and the second EC loop of claudin-1 (aa 144–161) were amplified using specific primers and subcloned into pGEX vector, and these GST fusion proteins were expressed in *E. coli* (BL21). GST fusion proteins with the first EC loop of claudin-3 were purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.5. Gel electrophoresis and immunoblotting

One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 12.5% gel) was performed based on the method of Laemmli [22]. Gels were stained with Coomassie brilliant blue R-250. For immunoblotting, proteins separated by SDS–PAGE were electrophoretically transferred onto nitrocellulose sheets, which were then incubated with primary antibodies. The antibodies were detected with a blotting detection kit (Amersham Pharmacia Biotech).

2.6. Overlay analyses

E. coli lysate or purified GST fusion proteins were separated by SDS–PAGE, and transferred onto nitrocellulose membranes. These membranes were treated with blocking solution (5% skim milk in TBS) overnight at 4°C, followed by a 16 h incubation with 10 μg/ml CPE in binding buffer (0.2 M phosphate buffer, pH 7.4, 100 mM NaCl, 0.2 mM NaN_3, 0.2 mM EGTA) at 4°C. After washing with the binding buffer several times, bound CPE was detected with anti-CPE pAb.

To determine the K_a value for the interaction between CPE and the second EC loop of claudin-3, the GST fusion protein with the second EC loop of claudin-3 on nitrocellulose membranes was incubated with various concentrations of purified C-CPE. The amounts of bound C-CPE were estimated by comparing the intensities of immunoblotted bands with those of various amounts of purified C-CPE on the same membranes using NIH image software, and Scatchard plots of the data were generated. Experiments were repeated three times for each estimation of K_a.

3. Results and discussion

We first compared the sensitivity to CPE of two L fibroblast transfectants, C1L and C3L cells, expressing exogenous claudin-1 and -3, respectively. As shown in previous studies [11,14,17], when purified CPE at 500 ng/ml was added into the culture medium followed by a 1 h incubation, C3L cells formed characteristic bleb balloons and underwent cell death (see Fig. 2b), whereas C1L cells did not exhibit any morphological changes up to 24 h incubation (see Fig. 2a). In this study, to determine the region responsible for the CPE sensitivity in claudin-3, we constructed two types of chimeric molecules between claudin-1 and -3 (Fig. 1A); claudin-1/3 containing the first EC loop of claudin-1 and the second EC loop of claudin-3, and claudin-3/1 containing the first EC loop of claudin-3 and the second EC loop of claudin-1. Then, we established L transfectants stably expressing claudin-1/3 or claudin-3/1 (C1/3L and C3/1L cells, respectively). Expression

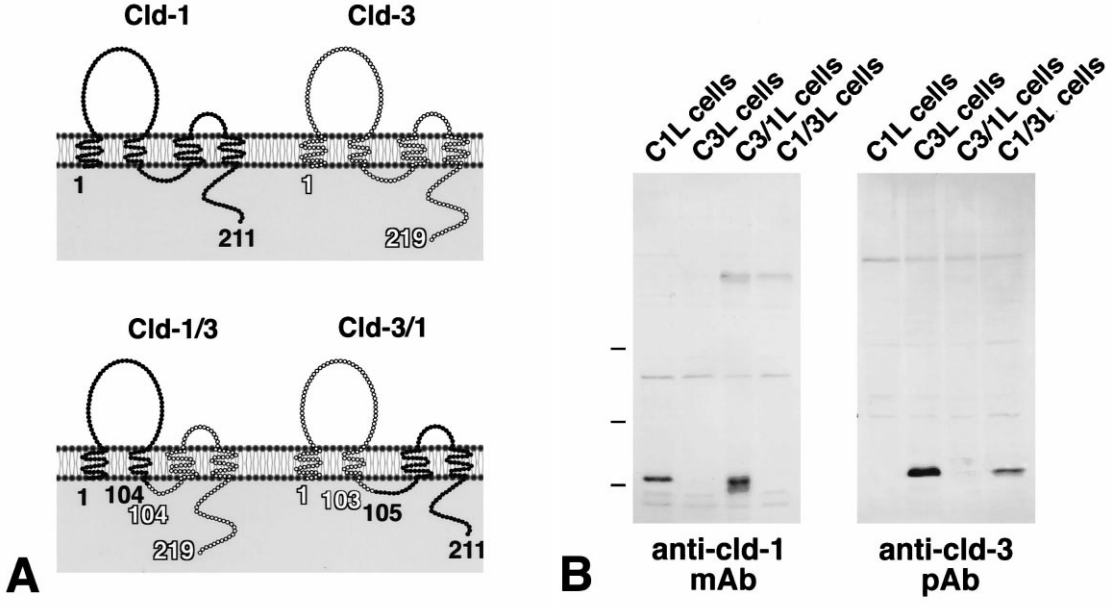

Fig. 1. Chimeras between claudin-1 and claudin-3. A: Structure of chimeric molecules. In claudin-1/3 (Cld-1/3), aa 1–104 of claudin-1 was fused with aa 104–219 of claudin-3, while claudin-3/1 (Cld-3/1) was composed of aa 1–103 of claudin-3 and aa 105–211 of claudin-1. B: Expression of chimeric molecules. Total cell lysates of L transfectants expressing claudin-1 (C1L cells), claudin-3 (C3L cells), claudin-3/1 (C3/1 cells) or claudin-1/3 (C1/3 cells) were separated by SDS–PAGE, followed by immunoblotting with anti-claudin-1 mAb or anti-claudin-3 pAb, which recognized the COOH-terminal cytoplasmic domains of the respective claudin species. Bars indicate molecular masses of 45, 31 and 21 kDa, respectively, from the top.

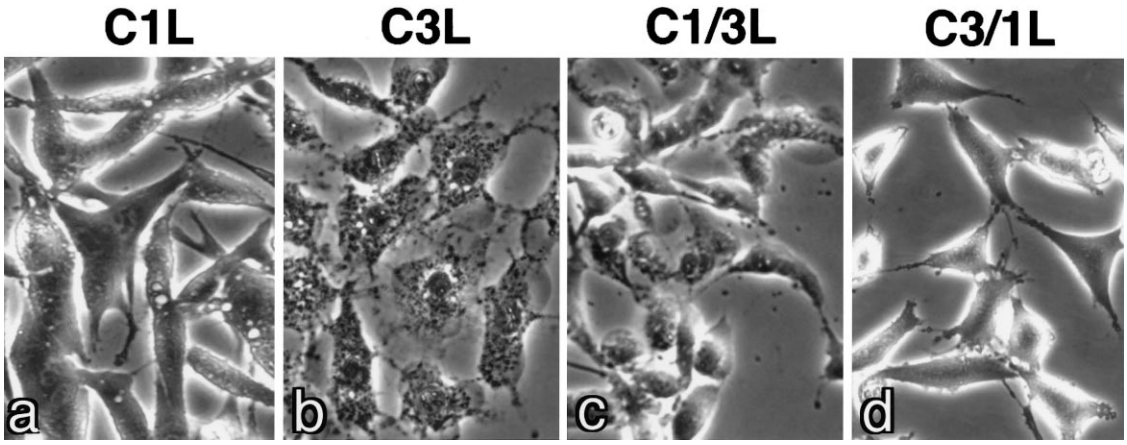

Fig. 2. Cytotoxicity of CPE on L transfectants. L transfectants expressing claudin-1 (C1L), claudin-3 (C3L), claudin-1/3 (C1/3L) or claudin-3/1 (C3/1L) were cultured in the presence of 500 ng/ml CPE. Phase-contrast microscopic images taken after 1 h incubation revealed that C3L (b) and C1/3L (c) cells, but not C1L (a) or C3/1L (d) cells, formed characteristic bleb balloons and underwent cell death.

of these chimeric molecules was confirmed by immunoblotting with anti-claudin-1 mAb and anti-claudin-3 pAb, which recognized the COOH-terminal cytoplasmic domains of the respective claudin species (Fig. 1B). As shown in Fig. 2, C1/3L cells, but not C3/1 cells, showed characteristic sensitivity to CPE, i.e. bleb formation and cell death. As C3L cells, but not C1L cells, were sensitive to CPE, these findings suggested that the interaction between the second EC loop of claudin-3 and CPE is required for the cytotoxicity of CPE.

Next, we examined the direct interaction between the second EC loop of claudin-3 and CPE. For this purpose, we performed overlay analyses. GST fusion proteins with the first EC loop of claudin-3, the second EC loop of claudin-3 and the second EC loop of claudin-1 were produced in *E. coli*, and these lysates were separated by SDS–PAGE and transferred onto nitrocellulose membranes. These membranes were incubated with CPE, and bound CPE was detected by anti-CPE pAb. As shown in Fig. 3A, CPE was detected on the band of the GST–second EC loop of claudin-3 fusion protein, but not on those of the GST–first EC loop of claudin-3 or GST–second EC loop of claudin-1. As CPE was not detected on the bands derived from *E. coli*, we concluded that CPE bound to the second EC loop of claudin-3 directly and specifically. Using this overlay system, we next quantitatively examined the binding affinity between the second EC loop of claudin-3 and the COOH-terminal half of CPE (C-CPE): the bands of GST–second EC loop of claudin-3 on nitrocellulose membranes were incubated with various concentrations of C-CPE, and

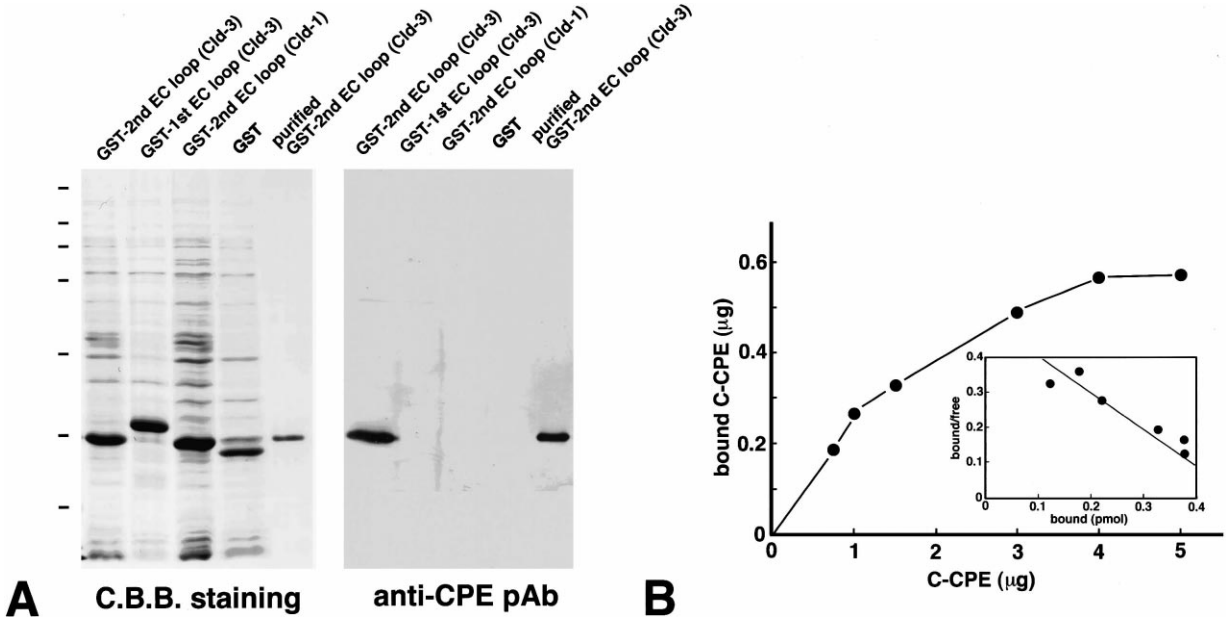

Fig. 3. Direct binding of CPE or C-CPE to the second EC loop of claudin-3. A: Overlay analysis. Crude lysates of *E. coli* expressing GST–second EC loop of claudin-3, GST–first EC loop of claudin-3, GST–second EC loop of claudin-1 or GST, and purified GST–second EC loop of claudin-3 were separated by SDS–PAGE (left panel), and transferred onto nitrocellulose membranes. These membranes were incubated with CPE and bound CPE was detected by anti-CPE pAb (right panel). CPE specifically bound to the second EC loop of claudin-3. Bars indicate molecular masses of 200, 116, 97, 66, 45, 31 and 21 kDa, respectively, from the top. B: Quantitative analysis of the binding between the second EC loop of claudin-3 and C-CPE. The GST fusion protein with the second EC loop of claudin-3 on nitrocellulose membranes (see A) was incubated with various concentrations of C-CPE and then the amount of bound C-CPE was estimated using anti-CPE pAb as described in Section 2. Scatchard plot analysis indicated that the K_a value was 1.0×10^8 M^{-1}.

then the amount of bound C-CPE on each band was quantified. Scatchard plot analysis indicated that the K_a value was 1.0×10^8 M^{-1} (Fig. 3B). Previously, using C3L cells and ^{125}I-CPE, we examined the binding kinetics of CPE to claudin-3, and calculated the K_a value as 8.4×10^7 M^{-1} [17]. Therefore, we concluded that the second EC loop is the site through which claudin-3 directly interacts with CPE on the cell surface.

Claudin-3 and -4, but not claudin-1 and -2, were reported to bind to CPE [17]. Questions naturally arose as to the binding affinity of the other claudin species to CPE. We examined the sensitivity to CPE of L transfectants expressing claudin-5, -6, -7, -8, -10 or -14 and found that claudin-6, -7, -8 and -14 but not claudin-5 and -10, bind to CPE at the K_a values of 9.7×10^7, 8.8×10^7, 1.0×10^6 and 3.6×10^6 M^{-1}, respectively. Further detailed mutagenesis analyses will clarify the relationship between the second EC loop amino acid sequences and their CPE-affinity in the future study.

The NH_2-terminal half of CPE was reported to increase membrane permeability by forming small pores in the plasma membrane [15,16]. Therefore, binding of the COOH-terminal half of CPE to specific claudins may facilitate pore formation by its NH_2-terminal half. As discussed previously [17], C-CPE can be utilized as a powerful tool to modulate the barrier function of TJs composed of CPE-sensitive claudin species. In this study, we showed that the second EC loop of CPE-sensitive claudins is responsible for the direct CPE/claudin interaction. Therefore, it would be possible to search for or synthesize polypeptides other than C-CPE that directly and specifically bind to the second EC loop of each claudin species. These polypeptides would provide not only powerful tools to modulate the TJ barrier function at the cellular level but also a new method for drug delivery at the whole body level.

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